

Rheumatoid blood decreases the adherence of polymorphonuclear cells (PMNs) to cultured endothelium

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SUMMARY Rheumatoid sera and plasma inhibited the adherence of normal blood polymorphonuclear cells (PMNs) to cultured porcine endothelium. This inhibition of adhesion was not seen when PMNs were treated with the plasma or serum from normal subjects or patients with other inflammatory arthropathies. The abrogation of PMN adherence was directly related to the levels of circulating immune complexes and was not dependent upon the type of anti-inflammatory therapy that the patients were receiving nor on any of the recorded clinical parameters. A similar inhibition of adhesion was seen with heat induced aggregated human IgG (HAGG) provided that serum was present in the culture medium. In view of these results we propose that circulating immune complexes in RA may have a significant role in controlling the interaction of PMNs with vascular endothelium and in perpetuating the entry of these cells into the synovial fluid of the inflamed joints.

Key words: rheumatoid arthritis, neutrophils, margination, immune complexes.

In the preceding paper it was shown that rheumatoid sera inhibited the adherence of normal blood polymorphonuclear cells (PMNs) to monolayers of porcine endothelium.¹ The adherence of normal PMNs to nylon fibre columns is reported to be enhanced when the cells are suspended in the plasma, but not the serum, of patients with acute inflammatory disease² and inhibited by the sera of patients receiving anti-inflammatory therapy.³ To characterise the inhibitory factor present in RA blood, samples of plasma and serum were prepared from patients with RA, whose clinical presentations were carefully recorded. Similar samples were obtained from patients with other inflammatory arthropathies and normal subjects. The adherence modifying activities of these plasma/serum samples were tested in a quantitative monolayer adhesion assay and screened for evidence of immunological abnormalities. After the observation that an inhibition of PMN adherence by the RA samples corre-

lated with their increased levels of circulating immune complexes further experiments were devised in which blood PMNs were cocultured with stable human IgG aggregates. When normal human serum was present in the assay these aggregates inhibited PMN adhesion, suggesting that immune complexes mediate the inhibitory activity present in the blood of patients with RA.

Patients and methods

PATIENTS

Sera and plasma were obtained from patients with RA attending the Rheumatology Department, The London Hospital. Aliquots of the samples were taken and stored at -70°C until use. Of the 26 patients with RA investigated, six were receiving prednisolone therapy in conjunction with non-steroidal anti-inflammatory drugs (NSAIDs), two penicillamine only, two gold and NSAIDs, two azathioprine and NSAIDs, and the remainder NSAIDs only. The disease activity of all the patients was assessed by an independent clinician. Blood samples were also obtained from three patients with

psoriatic arthritis, two with ankylosing spondylitis, and one with systemic lupus erythematosus (SLE); the patient with SLE was receiving prednisolone, chloroquine, and NSAIDs, and the other five patients NSAIDs only. In addition, samples of serum and plasma were obtained from 20 healthy hospital personnel. All rheumatoid samples were screened for latex titres, IgG, IgM, and IgA rheumatoid factor (RF) concentrations, immune complexes by the C1q binding and solid phase assay, and complement levels determined by measuring CH₅₀, C3, and C4.

ISOLATION OF PMNS

The method was a modification of that of Dioguardi *et al.*⁴ Fifty millilitres of heparinised blood (10 U/ml) were diluted with 350 ml of 0.83% ammonium chloride and allowed to stand for 10 min at room temperature. After centrifuging at 450 g for 10 min the supernatant was discarded and the NH₄Cl lysis stage repeated. The PMN pellet from the second lysis was washed three times by slow centrifugation (55 g for 10 min) in calcium and magnesium free Hanks's balanced salt solution (CMF-HBSS). This slow centrifugation sediments the large dense PMNs from the smaller less dense lymphocytes which remain in suspension. The absence of divalent cations in the medium prevented any spontaneous aggregation of the PMNs and greatly increased the yield of cells. The final cell pellet was adjusted to 1×10^6 cells/ml. Cell viability as shown by exclusion of the trypan blue was always >96%, and the purity of PMNs was approximately 90%.

This method was chosen because it was found to be more rigid than the dextran-Lymphoprep isolation technique used in the preceding paper. The adherence of PMNs was not modified by ammonium chloride treatment.

RADIOLABELLING OF PMNS WITH ⁵¹Cr

At the end of the last study¹ we showed that indium-111 (¹¹¹In) oxine itself augmented PMN adhesion to endothelial cells.⁵ Although this finding did not alter the interpretation of the results in the preceding paper,¹ PMNs in the present investigation were radiolabelled with ⁵¹Cr. This radiolabel does not enhance the adherence as does ¹¹¹In oxine. Preliminary studies showed radiolabelling to be most efficient when 100 µCi (3.7 MBq) ⁵¹Cr were incubated with 1×10^8 PMNs for one hour at 37°C with agitation every 15 min. The radiolabelled PMNs were washed on three occasions by suspending in 5 ml CMF-HBSS supplemented with 5% fetal calf serum and centrifuging at 450 g for 10 min.

The final cell pellet was resuspended in 5 ml EMEM (Eagle's minimum essential medium) sup-

plemented with 1 mM glutamine, 200 U/ml penicillin, and 100 U/ml streptomycin, buffered to pH 7.3 with 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) and filtered through 4 mm metal gauze to remove any large clumps. The viability of PMNs as assessed by trypan blue exclusion was >96%, and the cell concentration was adjusted to 1×10^6 cells/ml.

PREPARATION OF ENDOTHELIAL MONOLAYERS

The method was identical to that described in the preceding paper¹ except that the final seeding of endothelial cells in the multiwell plates was onto the floor of each chamber and not onto glass coverslips.

ADHERENCE ASSAY

Medium was aspirated from each well and the confluent endothelial monolayers washed twice with phosphate buffered saline (PBS) at 37°C to remove any contaminating fetal calf serum. To each well were added 100 µl of labelled PMNs (1×10^6 cells/ml) from normal healthy subjects, 200 µl of the

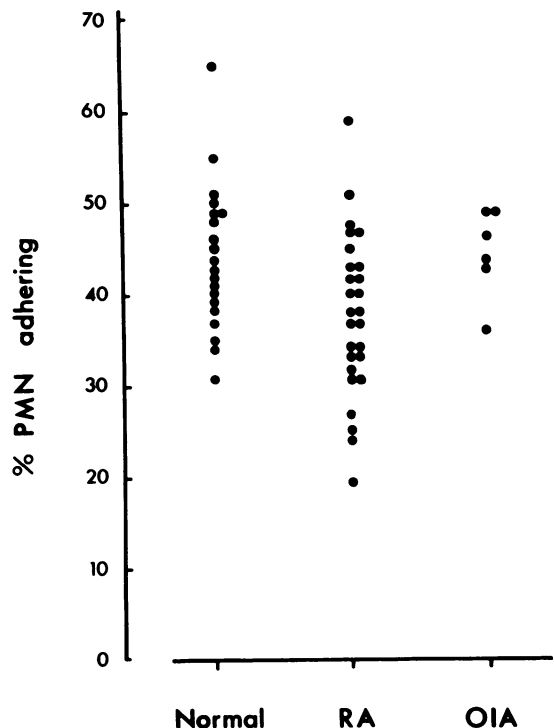


Fig. 1 Comparison of the effect of sera from normal subjects, patients with RA, and patients with other inflammatory arthropathies (OIA) on the adhesion of normal blood PMNs to cultured endothelium.

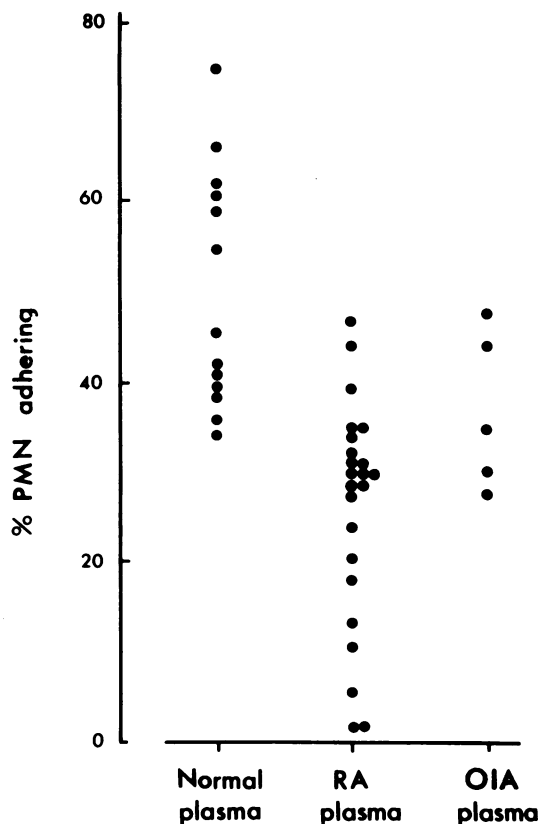


Fig. 2 Comparison of the effect of plasma from normal subjects, patients with RA, and patients with other inflammatory arthropathies (OIA) on the adhesion of normal blood PMNs to cultured endothelium.

test serum, and 700 μ l of EMEM. Each test was performed in triplicate in randomly allotted wells. After incubation at 37°C for 60 min the non-adherent PMNs were aspirated and the endothelial monolayers washed with PBS at 37°C to remove any loosely adherent cells. The endothelium was osmotically disrupted by treatment with 0.5 ml 0.1 M NaOH for 15 min. The lysate was aspirated, collected in tubes, and counted in a Packard auto-gamma scintillation counter.

The percentage of PMNs adhering to endothelium was calculated as follows:

$$\text{Adherence (\%)} = \frac{\text{cpm in each well} - \text{cpm background}}{\text{cpm of original PMN-cpm background suspension}} \times 100$$

PREPARATION OF STABLE HEAT INDUCED AGGREGATED HUMAN IgG (HAGG)
Aggregates of IgG were prepared as described

previously.⁶ Briefly, human IgG (Sigma) was dissolved in borate buffered saline (BBS) at a concentration of 30 mg/ml and clarified by centrifugation at 5000 rpm for 10 min. The supernatant was heated at 60°C until the absorbance at 40 nm reached 0.25 units, thereafter it was allowed to stand at 4°C for 48 h. The aggregates were selectively concentrated by polyethylene glycol (7%) precipitation, washed twice by centrifugation in BBS, and resuspended in 5 ml BBS. The HAGG was concentrated twofold by air drying in dialysis tubing to give a protein concentration of 2 mg/ml and diluted in EMEM (Gibco) for use as the test media in the adherence assay.

STATISTICAL METHODS

The data were parametric and the Student's *t* test was used to determine the significance of differences between the means of two groups.

Results

EFFECT OF RA SERUM AND PLASMA ON PMN ADHERENCE

The results, expressed as the percentage of PMNs adhering to endothelial cells, are shown in Fig. 1.

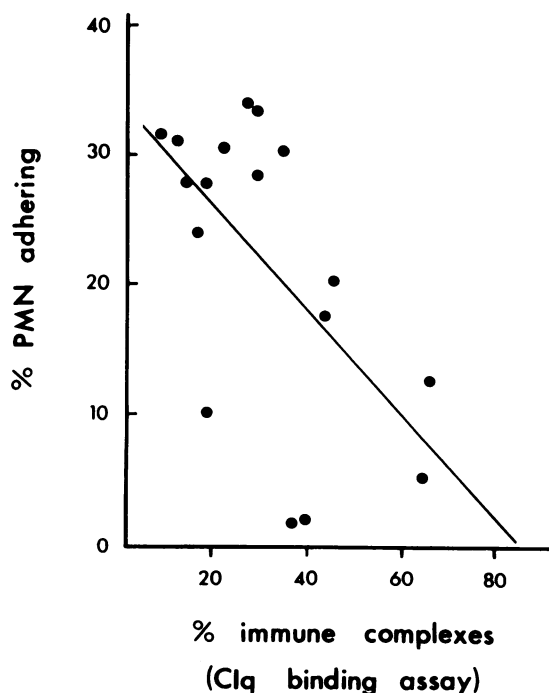


Fig. 3 The relation between the inhibition of PMN adhesion induced by the RA plasma and the level of circulating immune complexes as measured by the C1q binding assay.

Table 1 *Correlation between an inhibition of PMN adherence by RA plasma and various immunological parameters*

Parameter	r Value	Probability
Latex titre	-0.353	0.5>p>0.1
IgM RF	-0.333	0.5>p>0.1
IgA RF	-0.500	p<0.05*
IgG RF	-0.376	0.5>p>0.1
Total immune complexes		
by C1q binding assay	-0.597	p<0.01*
IgM C1q solid phase assay	-0.001	p>0.5
IgA C1q solid phase assay	-0.351	0.5>p>0.1
IgG C1q solid phase assay	-0.494	p<0.05*

*Significant negative correlations.

The mean (SD) percentage adherence for normal PMNs cocultured with 20% RA serum was 37 (10)%, which was lower ($p<0.01$) than the mean (45 (7)%) for PMNs cultured with serum from either normal subjects or patients with other inflammatory arthropathies (OIA).

When 20% plasma was used in place of serum this inhibition was more marked (Fig. 2). With RA plasma there was a mean 22 (11)% PMN adhesion in contrast with means of 38 (7)% and 48 (13)% PMN adhesion induced by plasma from subjects with OIA and normal subjects respectively. There was no significant difference between the effects of OIA and normal plasma on PMN adherence.

Linear regression analysis was used to determine if there was any relation between the extent of adherence inhibition induced by the RA plasma and the patients' clinical and immunological status. There were significant negative correlations between the inhibition of adherence and the level of immune complexes ($p<0.01$), as measured by the C1q binding assay (Fig. 3), and the level of IgA rheumatoid factor and immune complexes bearing IgG when measured by the C1q solid phase assay (Table 1). There was no correlation between the

extent of inhibition adherence and any of the other immunological measurements, patients' clinical features, or the type of anti-inflammatory therapy.

INFLUENCE OF SECRETORY PRODUCTS OF ENDOTHELIAL CELLS ON ADHERENCE

The secretion of cyclo-oxygenase derived metabolites of arachidonic acid, particularly prostacyclin, by endothelial cells is reported to inhibit the adherence of PMNs to endothelium.⁷ To investigate if the abrogation of adherence in the present study was owing to the RA plasma inducing the release of such metabolites from endothelium, monolayers of endothelial cells were pretreated with indomethacin (a standard cyclo-oxygenase antagonist) and the effect of the rheumatoid plasma on PMN adherence monitored. Plasma from three patients with RA induced a mean 33% inhibition of PMN adhesion to untreated endothelial cells, and 37% inhibition using endothelium that had been pretreated with 10 µg/ml indomethacin for 24 hours. Thus the inhibi-

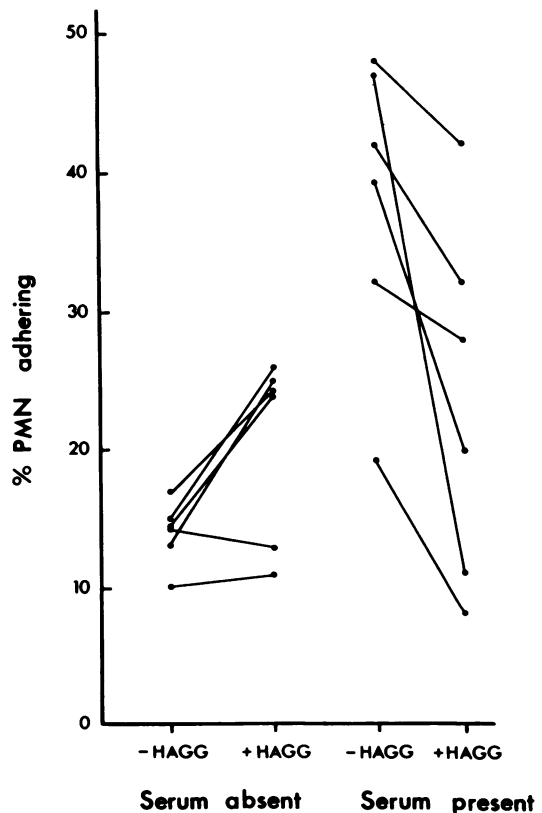


Fig. 4 Heat aggregated IgG (HAGG) modulation of PMN adherence in the absence and presence of autologous serum.

Table 2 *A comparison of the action of RA, OAI, and normal plasma on the adhesion of normal PMNs to monolayers of endothelial cells and serum coated plastic*

Plasma (n)	Percentage PMN adherence	
	Endothelium	Serum coated plastic
Normal (3)	46 (8)*	21 (9)
OIA (3)	43 (4)	14 (4)
RA (6)	30 (3)	8 (2)

*Values are mean (SD) percentage PMN adherence.

tory action of the RA plasma does not appear to be mediated via the release of cyclo-oxygenase products from endothelial cells.

To determine if RA plasma was inducing the release of other products of endothelial cells that might have been modifying the PMN attachment the action of plasma samples from six patients with RA, three patients with OIA, and three normal subjects on PMN adhesion was compared using endothelium and wells of plastic plates (Limbro) incubated with 20% heat inactivated fetal calf serum for one hour at 37°C and rinsed twice with PBS. The RA plasma induced a mean 35% ($p < 0.05$) and 62% ($p < 0.01$) inhibition of adhesion in the endothelium and serum coated plastic assay respectively when compared with the effect of plasma obtained from normal subjects and patients with OIA (Table 2). This experiment suggests that the inhibition of adhesion induced by the RA plasma was not mediated through the release of endothelial cell secretory products.

EFFECT OF IgG AGGREGATES ON PMN ADHERENCE

Since stable, heat induced aggregates of IgG (HAGG) are believed to share similar biological properties with immune complexes⁶ we investigated the action of such aggregates on PMN-endothelial cell adherence. In each of six experiments 100 µg of HAGG was preincubated with 1×10^6 labelled PMNs for 30 min at 37°C. Thereafter, aliquots of the PMN suspension were added to the endothelial cell monolayers. When serum was absent from the culture medium the HAGG induced a mean 33% increase in adhesion (Fig. 4). In contrast, when 20% autologous sera were added to the assay the HAGG induced a mean 28% inhibition of adhesion.

Discussion

The demonstration that serum and particularly plasma from patients with RA inhibited the adhesion of normal blood PMNs to cultured endothelium repeats the findings of the preceding paper. This inhibition, which was not related to duration of disease, disease activity, or extravascular manifestations, was directly correlated with the level of circulating immune complexes as measured by the C1q binding assay.

An inhibitor of PMN adherence to nylon fibres has been described in the plasma (but not the serum) of patients receiving non-steroidal anti-inflammatory drugs.³ Such a drug related factor seems unlikely to be responsible for the inhibition noted in the present study since plasma from the patients with OIA, who were also receiving NSAIDs,

did not modify PMN adhesion. Moreover, there was no correlation between the type of anti-inflammatory therapy and the degree of inhibition, and recent studies in our laboratory show that the incubation of PMNs with NSAIDs *in vitro* does not modify their adherence to cultured endothelium (manuscript in preparation).

The attachment of PMNs to endothelium is enhanced by complement.⁸ All RA samples tested, however, possessed complement activities that were within the normal range.

A major secretory product of endothelial cells is prostacyclin,⁹ which decreases PMN adherence to cultured endothelium⁷ and nylon.¹⁰ Its synthesis and release by endothelial cells is enhanced by serum,¹¹ hydrogen peroxide,^{12, 13} and leucotrienes.¹⁴ Because the inhibitory activity of the RA plasma was not modified when the endothelial cell monolayers were pretreated with indomethacin we infer that the secretion of cyclo-oxygenase products, either directly or indirectly, by the release of degranulation products from activated PMNs is not involved.

The abrogatory activity induced by the RA serum/plasma appears to be due in part to the level of immune complexes containing IgG, a conclusion that is supported by the demonstration that IgG aggregates also impeded PMN adhesion. Hashimoto and Hurd reported that high concentrations of IgG aggregates and immune complexes increased the attachment of PMNs to endothelium when serum was absent from the coculture¹⁵; a finding that we support. As shown in the present report, however, when serum is included in the assay the IgG aggregates induced a decrease in PMN adhesion. We therefore propose that *in vivo* certain circulating immune complexes restrict PMN attachment to vascular endothelium. Our preliminary data suggest that this inhibition is mediated via complement. Immune complexes may bind to the Fc and C3b receptors of the PMNs and mask surface ionogenic groups, whose expression is necessary for the cell's interaction with endothelial cells. Experiments are in progress to determine if the membranes of PMNs treated with immune complexes are still accessible to the binding of antibodies directed against such determinants.

At first, an abrogation of PMN interaction with endothelial cells by immune complexes appears to contravene the clinical observations of a large persistent infiltration of RA blood PMNs into the joint synovial fluid. We speculate, however, that immune complexes, by restricting the number of PMNs adhering to endothelium of the general vasculature, increase the number of cells available to synovial endothelial cells, whose distinct membrane phenotypes facilitate the margination of

PMNs bearing immune complexes. Such a mechanism may be implicated in the increased susceptibility of patients with RA to infection, and be relevant to the inflammatory recruitment of other leucocytes. We are currently investigating this possibility.

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